The effects of chronic exposure to produced water on growth and hepatic ethoxyresorufin *O*-deethylase (EROD) activity of juvenile Atlantic cod (*Gadus morhua*)

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by

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ABSTRACT

Lyons, M.C., Wong, D.K.H., MacKeigan, K., Burridge, L.E., Lee, K. and Robinson, B. 2014. The effects of chronic exposure to produced water on growth and hepatic ethoxyresorufin *O*-deethylase (EROD) activity of juvenile Atlantic cod (*Gadus morhua*). Can. Tech. Rep. Fish. Aquat. Sci. 3101: v + 20p.

The discharge of produced water (PW) from offshore oil and gas facilities remains an environmental concern due to uncertainty regarding its fate, transport and potential biological effects. This oil-related effluent containing polycyclic aromatic hydrocarbons (PAHs), phenols, alkylphenols (AP) and heavy metals is rapidly diluted after discharge into the open ocean. Marine fish may be chronically exposed to diluted PW at distances far from oil and gas production sites. Juvenile Atlantic cod were exposed intermittently over a period of 14 weeks to 0, 100 or 200 ppm of PW effluent from various oil and gas installations in Atlantic Canada, including the Sea Rose and Terra Nova floating production, storage and offloading vessels (FPSO), and the Venture and Thebaud gas production platforms. Three times weekly the fish were fed and then exposed to PW. PAH exposure was measured by induction of cytochrome P-450 (CYP1A) as indicated by EROD activity. Growth and food consumption were monitored. At the end of the exposure there were no significant effects of PW observed on EROD activity. Significant effects on specific growth rate (SGR) were seen in fish exposed to 200 ppm Sea Rose PW ($P< 0.05$) and 100 ppm Thebaud PW ($P< 0.005$) but there was no observed dose response. The results suggest that intermittent exposure to environmentally relevant concentrations of PW from some oil and gas platforms can affect growth.

RÉSUMÉ

Lyons, M.C., Wong, D.K.H., MacKeigan, K., Burridge, L.E., Lee, K. and Robinson, B. 2014. The effects of chronic exposure to produced water on growth and hepatic ethoxyresorufin *O*-deethylase (EROD) activity of juvenile Atlantic cod (*Gadus morhua*). Can. Tech. Rep. Fish. Aquat. Sci. 3101: v + 20p.

Le déversement d'eau produite depuis les installations pétrolières et gazières en mer reste une préoccupation environnementale en raison des incertitudes relatives au devenir de cette eau, à son transport et à ses éventuels effets biologiques. Cet effluent pétrolier contenant des hydrocarbures aromatiques polycycliques, des phénols, des alkylphénols et des métaux lourds se dissout rapidement après son déversement en haute mer. Il est possible que les poissons marins soient chroniquement exposés à l'eau produite diluée, même dans des endroits éloignés des sites de production pétroliers et gaziers. Des morues franches juvéniles ont été exposées de façon intermittente sur une période de 14 semaines à de l'eau produite diluée à 100 et 200 ppm et venant de plusieurs installations pétrolières et gazières du Canada atlantique, dont les unités flottantes de production, stockage et déchargement en mer Terra Nova et Sea Rose, et les plateformes de production gazière Venture et Thebaud. Les poissons étaient nourris, puis exposés à l'eau produite trois fois par semaine. L'exposition aux hydrocarbures aromatiques polycycliques était mesurée par l'induction de cytochrome P450 (CYP1A) comme indiqué par l'activité de l'enzyme EROD. La croissance et la consommation de nourriture étaient contrôlées. À la suite de l'exposition, aucun effet significatif de l'eau produite n'a été constaté sur l'activité de l'enzyme EROD. Des effets significatifs ont en revanche été constatés sur le taux de croissance spécifique chez les poissons exposés à l'eau produite de Sea Rose à 200 ppm ($p < 0,05$) et à l'eau produite de Thebaud à 100 ppm ($p < 0,005$), mais aucune réaction à la dose n'a été observée. Ces résultats laissent entendre qu'une exposition intermittente de poissons à l'eau produite de certaines plateformes pétrolières et gazière à des concentrations pouvant se trouver dans l'environnement peut affecter leur croissance.

INTRODUCTION

Produced water (PW) (formation and injected water containing production chemicals) represents the largest volume waste stream in oil and gas production operations on most offshore platforms. There is considerable concern about the ocean disposal of PW, because of the potential danger of chronic exposure resulting in ecological harm (Neff et al, 2011). With anticipated increases in the number of new offshore platforms, PW discharge has been identified as an issue of concern by both regulators and environmental groups (Zhao et al., 2008). The chemical characteristics of PW are different for each production platform or formation from which the oil is extracted. It is typically highly saline and contains elevated levels of heavy metals, hydrocarbons (including polycyclic aromatic hydrocarbons (PAHs)), alkylphenols (AP), ammonia and radionuclides compared to the receiving environment (Lee et al., 2005; Burridge et al., 2011). The physical and chemical properties of PW vary widely depending on the geologic age, depth, and geochemistry of the hydrocarbon-bearing formation, as well as the chemical composition of the oil and gas phases in the reservoir, and process chemicals added during production. Because no two PWs are alike, region specific studies are needed to address the environmental risks from its discharge (Neff et al., 2011).

Although the acute toxicity of PW discharges into the environment for marine organisms is probably a threat only within the direct zone of the discharge, continual chronic exposure may cause sub-lethal changes in populations and communities, including decreased community and genetic diversity, lower reproductive success, decreased growth and fecundity, respiratory problems, behavioral and physiological disorders, decreased developmental success and endocrine disruption (Neff et al., 2011). One of the most toxic groups of compounds found in PW, PAHs has been shown to have cytotoxic, immunotoxic, mutagenic or carcinogenic effects in marine organisms. Ethoxyresorufin *O*-deethylase (EROD) is a cytochrome enzyme induced in the liver of fish in the presence of PAHs. Hepatic EROD activity is a good biomarker of PAH exposure (Burridge et al., 2011). Change in weight (mass) is the most commonly used assessment for growth performance. When growth rate is exponential, as it usually is over intervals of a year or less, growth can be expressed as an instantaneous growth rate or as a percentage of instantaneous growth called specific growth rate (SGR) (Busacker et al., 1990). Food intake (FI) and gain in fish mass are used to calculate feed conversion efficiency (FCE) which is a measure of the ability of an organism to convert ingested food into new tissue and is dependent on the quantity and quality of food available and the environmental conditions of the habitat (Mateo, 2007).

Atlantic cod (*Gadus morhua*) is a species of considerable ecological and economic importance for Canada. The areas of the northwest Atlantic that have the highest abundance of Atlantic cod are in Canadian waters and include the eastern coast of Labrador south of Cape Harrison, off eastern Newfoundland, the Flemish Cap, the Grand Bank, the Gulf of St. Lawrence, and the Scotian Shelf (Lough, 2004). All four of the PWs used in this study are discharged into Canadian waters where Atlantic cod may be exposed. By day young juvenile cod remain on the bottom, but at night they rise several meters into the water column and drift in the tidal current while feeding (Lough, 2004).

Examining the chronic effects of PW on cod growth and feeding will shed light on the potential long-term impact of oil and gas offshore developments on stocks (Hamoutene et al., 2011). Metabolic capacities, feeding and digestive physiology of fish are influenced by environmental parameters. Chronic exposure to PW might potentially affect feeding, which in turn might have consequences on growth/health status of fish populations (Hamoutene et al., 2011).

In this study we intermittently exposed juvenile Atlantic cod to two concentrations of PW and assessed potential effects on hepatic EROD activity and growth parameters while monitoring food consumption. The fish were individually tagged with Passive Integrated Transponders (PIT) so that growth could be monitored three times over the fourteen week exposure. Intermittent exposure to PW concentrations of 100 ppm (10 000 x dilution) and 200 ppm (5000 x dilution) was used in an attempt to mimic the rapid dilution taking place after release in the environment. Rapid dilution of PW by at least 240 x has been shown to occur within 50-100 m, 1000 x by 4-5 km and up to 9000 x at 20 km from the discharge site (Somerville et al., 1987; Murray-Smith et al., 1996; Pérez-Casanova et al., 2010). Factors that affect the rate of dilution of PW include discharge rate and height above or below the sea surface, ambient current speed, turbulent mixing regime, water column stratification, water depth, and differences in density and chemical composition between the PW and ambient seawater (Neff et al., 2011; Reed and Rye, 2011; Niu et al., 2011).

MATERIALS AND METHODS

PRODUCED WATER COLLECTION

Two of the PWs used in this study were from the gas platforms, Venture and Thebaud. These platforms are off the coast of Nova Scotia, Canada, in the Scotian Shelf region of the Atlantic Ocean (Fig. 1). The other two PWs studied were from White Rose and Terra Nova oil fields (Fig. 1). Sea Rose is a floating production storage and offloading vessel (FPSO) located in the White Rose oil and gas field, off the coast of Newfoundland, Canada on the eastern edge of the Grand Banks in the North Atlantic Ocean. Terra Nova is a FPSO located south of White Rose. Samples of PW were collected in acid-washed (1 M HCl) nalgene high-density polyethylene jerricans that were provided to platform staff for the collection of PW. Instructions for collection were to fill the jerricans to overflowing to eliminate any headspace. Once collected, the PW samples were immediately delivered to DFO staff and the containers were sealed with electrical tape, refrigerated and transported to the Bedford Institute of Oceanography, Dartmouth, Nova Scotia and then on to St. Andrews Biological Station, St. Andrews, New Brunswick where they were refrigerated at 4°C for the duration of the study.

FISH STOCKS

The juvenile Atlantic cod (*Gadus morhua*) used in the 2010 experiment were grown from eggs as part of ongoing research projects at the St. Andrews Biological Station (SABS), St. Andrews, New Brunswick, Canada. Juvenile cod were obtained in 2011 and 2012 from Great Bay Aquaculture, Portsmouth, New Hampshire, USA. The fish were held in flow through filtered sea water (Passamaquoddy Bay, New Brunswick, Canada) at

ambient temperature and under simulated natural photoperiod for July and August. Dissolved oxygen and water temperature were recorded daily. The fish were manually fed once daily with Europa, 2.0 mm sinking feed from Skretting North America (Bayside, New Brunswick, Canada). Initially the fish in each tank were slowly hand fed an amount of food equal to 3% to 4% of the estimated total mass of fish in the tank. Each tank bottom was fitted with a removable central standpipe that allowed water to drain freely but that could be lifted daily to allow feces and uneaten food to be flushed down the drain. The juvenile cod were anaesthetized with tricaine methanesulfonate (MS222; Syndel Laboratories Ltd, Vancouver, BC, Canada), PIT tagged and length and weight were recorded for each fish so that growth of the individual fish could be followed throughout the study. Groups of forty fish were placed in 400 L round fibreglass tanks with flow through filtered seawater delivered from a common header tank. Water was delivered at the water surface through tygon tubing. Water exited the tank through holes at the bottom of the central stand pipes. Initial fish weights were 9.4 ± 4.0 g (mean ± SD) in 2010, 13.1 ± 2.8 g in 2011 and 14.0 ± 2.2 g in 2012. The fish were allowed to recover in ambient seawater for 4 weeks in 2010, 2 weeks in 2011 and 3 weeks in 2012 before chronic exposures were started. At the end of the acclimation period, tanks were assigned to treatments: 2 control (0 ppm) tanks (n=40, n=40) and 2 treatment tanks – a 100 ppm dose (n=40) and a 200 ppm dose (n=40).

FISH EXPOSURE

The fish were held in 250 L of seawater and the water flow was maintained at 2.5 $L \cdot min^{-1}$ so that the pulse dose of PW was gradually diluted over a period of ~8 h. Prior to each feeding, standpipes were briefly lifted in each tank to remove feces from the bottoms of the tanks. Three times a week, for fourteen weeks the fish in each tank were manually fed a preweighed amount of food. The amount of preweighed food was kept consistent between tanks but adjusted periodically so that the cod were always fed beyond apparent satiation and there was always an excess amount of food on the bottom of the tank to collect. Immediately after feeding, the 100 ppm (10 000 x dilution) and the 200 ppm (5000 x dilution) groups were exposed to one pulse of PW from Sea Rose, Terra Nova, Thebaud or Venture platforms. The PW, either 25 mL (10 000 x dilution) or 50 mL (5000 x dilution) was poured slowly from a graduated cylinder onto the water surface. After four hours the standpipes were briefly lifted in each tank and the uneaten food was allowed to go down the drain to be collected in a net. The uneaten food was placed in a pre-weighed aluminum pan and allowed to air dry for five days in a fume hood before weighing. Five days of air drying allowed the food to be dried to constant mass. Ambient water temperature followed a similar trend for the fourteen week periods in 2010, 2011 and 2012 with highs in September of 13.7°C, 14.0°C and 14.8°C, respectively at the beginning of the exposures to lows in December of 7.4°C, 7.6°C and 7.4°C, respectively at the end of the 14 weeks. Due to construction and demolition activities at SABS over the three years, experiments were performed in three different laboratories but experimental conditions (tank size, water flows and photoperiod) were kept as consistent as possible.

SAMPLING

At five, ten and fourteen weeks, five fish per tank were sacrificed. Fish were stunned with a sharp blow to the head. Livers were excised, weighed, placed in foil, frozen in liquid nitrogen and stored in a -80 ℃ freezer until EROD analysis was performed. Length, weight and sex were recorded for each fish. Remaining fish were anaesthetized, scanned for PIT tag, length, weight and liver weight were recorded and then the fish were allowed to recover. Feed intake (FI) and feed conversion efficiency (FCE) were calculated at the five, ten and fourteen week sampling time points for each treatment tank (n=1, data of the 2 controls combined). The specific growth rates (SGR) of all fish were calculated for the three time periods: PIT tag date to five week sampling date, five to ten week sampling date and ten to fourteen week sampling date. Daily food intake (DFI) was calculated as:

$$DFI\ (g \cdot day^{-1}) = g\ feed\ into\ tank - g\ uneaten\ feed$$

FI and FCE for each treatment were calculated for the 5 week, 10 week and 14 week periods as:

$$FI = total\ of\ DFI\ (g, dry\ weight) \times total\ biomass\ (g)^{-1}$$

$$FCE = gain\ in\ fish\ mass \times g\ feed\ consumed^{-1}$$

The SGR was calculated for individual fish using the following formula:

$SGR = \left((\ln Y_2 - \ln Y_1) \times (t_2 - t_1)^{-1}\right) \times 100$ where Y_2 is the weight at sampling, Y_1 is the weight at the previous sampling , t_2 is the day at sampling and t_1 is the day at the previous sampling.

EROD ASSAY

Frozen livers were thawed, a sub-sample taken, put in a tared micro-centrifuge tube and weight recorded. Sub-samples of liver were homogenized and diluted with HEPES-KCl buffer (pH 7.5, 0.15 M KCl, 0.02 M HEPES) using a hand-held motor driven Kontes pestle. All steps were carried out on ice. The homogenates were centrifuged at 9850 rpm for 20 minutes at 2 °C. The post-mitochondrial supernatant (S-9 fraction) was collected with a Pasteur pipet, taking care to avoid the pellet and the floating lipid layer (Hodson et al., 1991). S-9 fractions were frozen at -80°C in micro-centrifuge tubes until analysis.

The kinetic microplate assay used is described in Hodson et al. (1996). EROD activity in cod liver S9 fractions was measured using a BioTek FLx 800 spectrofluorometer using excitation and emission wavelengths of 530 nm and 590 nm respectively. Plots of fluorescence versus time were created and the linear portions of the curves were selected for calculating slopes. The protein levels in the same S-9 samples were quantified with a Bio-Rad assay (Bradford) dye reagent using a BioTek Powerwave XS UV/Vis spectrophotometer with absorbance set at 600 nm. BioTek Gen 5 software was used to view triplicate results for each sample. EROD activity in juvenile cod was calculated from the slope of the curve over the selected time. Activity was converted to picomoles resorufin per minute per milligram protein by using the slope of the resorufin standard curve and the protein concentration of the sample from the protein assay.

CHEMICAL ANALYSIS

PW collected from Sea Rose, Terra Nova, Thebaud and Venture platforms was analysed at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia. All PW samples used in this study were analysed for a suite of organic and inorganic constituents, and the analytical methods used for analysis are summarized elsewhere (Lee et al., 2011). The

time between sample collection and analysis varied, sometimes as short as a few weeks, other times 8-12 months. PWs that could not be analysed within a few weeks of collection were subsampled and preserved with HCl so losses due to degradation would have been minimal.

STATISTICAL ANALYSIS

EROD activities were log- transformed to establish normal distribution and homogenous variances. Log-transformed data were treated by analysis of variance (level of significance was $P < 0.05$) to determine if differences were present between treated and untreated fish. Levene's test was used to test for homogeneity of variances and the normality of the SGR data was evaluated using the Shapiro-Wilk test. Data that were normally distributed were analysed for treatment effect using a 1-way ANOVA at each sampling day. If transformations did not correct non-normal distributions data were analysed for treatment effect using the Kruskal-Wallis test at each sampling day. Dunnett's post hoc comparison test was used to compare the SGR of the control fish with those of treated fish if equal variances were assumed and Dunnett T3 test was used if equal variances were not assumed. Data sets for the 2 control tanks were combined each year after analysing for tank effect and finding no significant difference ($P< 0.05$). Since FI and FCE were calculated per treatment and therefore n=1, no statistical analysis were conducted on these parameters however values are included for information purposes.
SPSS SAS version 19.0 (SAS Institute Inc., Cary, NC, USA) was used to conduct all of the statistical analyses.

RESULTS

EROD ACTIVITY

There were no significant ($P < 0.05$) differences in hepatic EROD activity between control and treated fish (Sea Rose, Thebaud and Venture PW) at any of the sampling times. Due to elimination of the contaminants program by DFO, EROD analysis was not performed for the fish exposed to Terra Nova PW. The mean EROD activity (mean±SE) in control fish ranged from 2.873±0.540 pmol·min^{-1}·mg^{-1} to 4.774±1.191 pmol·min^{-1}·mg^{-1} over the 14 week exposures. The mean EROD activity in treated fish ranged from 1.687±0.425 pmol·min^{-1}·mg^{-1} to 6.846±2.736 pmol·min^{-1}·mg^{-1} (Appendix 1, Table 1).

GROWTH PARAMETERS AND FOOD INTAKE

Comparisons between initial weights of fish showed that there were significant differences between years but there were no differences between control fish and treated fish initial weights for individual years (data not shown). The FI of the treated fish was similar to control fish for each of the sampling times each year (Figure 2 & Appendix 1, Table 2). The FI results were calculated based on total mass of fish in the treatment tanks at sampling time and are therefore corrected for mortality. The FCE of treated fish was similar to control fish for each of the sampling times with two exceptions - the 100 ppm Thebaud PW treated fish FCE was 32% lower than the control fish FCE and the 100 ppm Venture treated fish FCE was 30% higher than the control fish FCE in the final third of the study. FCE data is presented for the final third of the exposure period in Appendix 1, Table 2. Comparison of the mean SGR (mean±SD) of control and treated juvenile cod

showed that mean SGR of treated fish were significantly different from controls only in the final third of the exposure period (weeks 10 to 14) for two treatments -100 ppm Thebaud PW (Dunnett's $P<0.005$) and 200 ppm Sea Rose PW (Dunnett's $P<0.05$) (Appendix 1, Table 3). There were no effects on SGR seen at the 14 week time point for the 100 ppm Sea Rose PW and 200 ppm Thebaud PW treatments. There were no significant differences ($P < 0.05$) between mean SGR of treated fish and control fish in the first ten weeks of the study (Appendix 1, Table 3).

There was some loss of fish during the studies. Mortality from unknown causes throughout the studies was unrelated to treatment (2010- 15%, 2011- 2.5% and 2012- 1%). Juvenile cod are known to be cannibalistic and we saw evidence of this in the gut contents of some fish when sampling in 2010. Other sources of fish loss in 2010 were failure to recover from anesthetic after length and weights were recorded at sampling times, fish presumed lost down the drain when standpipes were lifted to remove feces prior to feedings and fish that were sacrificed after PIT tags failed to scan at sampling times. Greater care was taken in 2011 and 2012 to ensure fish were graded for size (> 10 g and < 20 g) at the beginning of the study so as to minimalize cannibalism and loss of fish down drains.

CHEMICAL ANALYSIS

A summary of the PW organic components is presented in Appendix 2, Table 1. Inorganic components are listed in Appendix 2, Table 2 with the concentrations of total metals (dissolved + particulate phase) in the PWs. The concentration of sulphur in Sea Rose and Terra Nova PW was 3 orders of magnitude greater than Venture and Thebaud. Conversely, both Venture and Thebaud had concentrations of barium, iron, lead, manganese, strontium and zinc that were 2-3 orders of magnitude greater than Sea Rose and Terra Nova. Compared to metals, the concentrations of organic constituents in the four PWs varied by an order of magnitude or less (Appendix 2, Table 1). Concentrations of BTEX (Benzene, Toluene, Ethyl benzene, Xylenes) ranged between 8.8 and 15.4 $mg \cdot L^{-1}$ (Appendix 2, Table 1). Concentrations of PAHs were very similar between the four PW samples, with total PAH (parent + alkylated) concentrations ranging between 0.39 and 0.88 $mg \cdot L^{-1}$ (Appendix 2, Table 1). High molecular weight PAH compounds with $\geq$4 benzene rings were detected in the Venture, Sea Rose and Terra Nova PWs with total concentrations up to 0.03 $mg \cdot L^{-1}$, while they were not detected in the Thebaud sample (data not shown). A wide range of phenolic compounds were also detected (data not shown), with total phenol concentrations higher in the gas production well PWs (10.6 and 16.5 $mg \cdot L^{-1}$ for Venture and Thebaud, respectively) versus the oil production wells (3.5 and 4.0 $mg \cdot L^{-1}$ for Sea Rose and Terra Nova, respectively) (Appendix 2, Table 1). Straight chain saturated hydrocarbons (alkanes) were also detected between 0.32 and 1.69 $mg \cdot L^{-1}$ in the four PWs (Appendix 2, Table 1).

DISCUSSION

The hepatic EROD activity of the juvenile Atlantic cod did not respond to the chronic exposures of PW indicating that the dilute exposure concentrations of PW used in this study contained insufficient concentrations of PAHs to affect hepatic EROD activity.

Burridge et al. (2011) measured PAHs in raw Hibernia PW but were unable to detect PAHs in water samples from their chronic exposure. They reported a maximum measured hepatic EROD value of 5 $pmol \cdot min^{-1} \cdot mg^{-1}$ during a 45 day exposure of juvenile cod to 0.05% Hibernia PW and did not see a significant difference in EROD activity between treated and untreated fish. In the present study hepatic EROD activities for juvenile cod were in the same range of values previously reported in this species (Aas et al., 2000; Burridge et al., 2011; Lyons et al., 2011; Nahrgang et al., 2013). Although EROD activity has been a sensitive biomarker in Atlantic cod exposed to PAHs it may not be a reliable indicator in chronic intermittent exposures of PW at low concentrations. Lie et al. (2009) saw significant up-regulation gene coding for CYP1A in a low AP exposure group of Atlantic cod but not in cod exposed to high AP or PW. Furthermore, Holth et al. (2009) suggested that CYP1A activity (EROD) appears to be sensitive to the influence of antagonists such as some PAHs and APs and adaption.

The SGR values were in the same range of values previously reported for Atlantic cod by other investigators (Imsland et al., 2005; Pérez-Casanova et al., 2009; Burridge et al., 2011). Mean SGR values generally decreased over the 14 weeks for control and treated fish probably due to the drop in ambient water temperature from ~14°C to ~7°C from September to December. Colder temperatures have a significant negative impact on fish appetite, growth and activity (Pérez-Casanova et al., 2009). In this study effects were seen on mean SGR of juvenile cod exposed intermittently to 100 ppm (10 000 x dilution) Thebaud PW and 200 ppm (5000 X dilution) Sea Rose PW when compared to controls and effects were only seen in the final third of the 14 week study. However, cod exposed to 200 ppm (5000 x dilution) Thebaud PW did not have significantly lower mean SGRs in the final third of the study indicating a lack of dose response. There is very little difference in mean SGR between the 100 ppm and 200 ppm PW treated groups at any of the sampling times except for two affected treatments (Appendix 1, Table 3). Perhaps the doses used in this study are near the lower limit of the range in which effects can be seen. The relationships between dose and response have been unclear in other studies suggesting that there could be a threshold level for effects of AP exposure on reproduction (Holth et al., 2010). Meier et al. (2007) found low doses produced the same decrease in 17β-estradiol or 11-ketoestosterone levels as higher doses. Lie et al. (2009) saw even more pronounced differences in gonadal somatic index and vitellogenin in fish exposed to low AP concentrations than in fish exposed to high concentrations. Stephens et al. (2000) used a semi-static exposure of post-metamorphic juvenile turbot, *Scophthalmus maximus* to PW from a North Sea oil platform. They saw no effect on SGR after 1 or 3 week exposures but did see significant effects in the growth of turbot exposed to 0.001% PW (100 000 x dilution) after 6 weeks which the authors attributed to an increase in energy expenditure due to increased swimming activity. Increased swimming activity in the wild may be an avoidance response leading to movement out of an effluent plume. This was their lowest exposure concentration and they did not see significant growth effects at any of the higher exposure concentrations. In contrast, they saw reduced swimming activity in the fish exposed to 1% PW (100 x dilution) and suggested narcotic action of either the external hydrocarbons or those accumulated internally. Stephens et al. (2000) also suggested that they could not presume growth would be unaffected in the

wild. Reduced physical activity can be predicted to reduce food-searching time and capture resulting in negative impact on growth.

There are some studies that address the potential effect of PW on the growth parameters of economically important finfish species. Pérez-Casanova et al. (2010) exposed juvenile cod intermittently for 22 weeks to Hibernia platform PW at 100 ppm and 200 ppm and saw no effects on growth or food intake. Hamoutene et al. (2011) exposed larger cod (average weight 774 g) intermittently for 11 weeks to Hibernia platform PW at the same concentrations and saw no effects on growth or food intake. Burridge et al. (2011) continuously exposed juvenile cod (mean weight 23 g) to 0.05% Hibernia PW (2000 x dilution) for 45 days and saw no growth effects. Pérez-Casanova et al. (2012) exposed Atlantic cod intermittently for 8 weeks to the aqueous fraction of Hibernia PW at high dose (1000 x dilution) and low dose (10000 x dilution) and saw no effects on growth or food consumption.

Fish in this study were provided with a limited body-weight related ration of food to ensure that they were hungry and increase the chances that they would feed. The rations offered to the fish were adjusted over the 14 week exposure period and were not consistent from year to year. The amount of preweighed food was adjusted periodically so that the cod were always fed beyond apparent satiation and there was always an excess amount of food on the bottom of the tank to collect. Comparisons of food intake from year to year were not made since food rations were not kept consistent from year to year. The FI values were normalized with the body mass of the fish for each treatment tank and FI values for fish exposed to PW were similar to the FI values for control fish. This indicates that the reduced mean SGR of the 100 ppm Thebaud PW and 200 ppm Sea Rose PW exposed fish in the final third of the study were not a consequence of reduced food intake. It may be worth noting that the FCE value of the 100 ppm Thebaud PW treatment group was 68% of the control fish FCE value for the final third of the study. The FCE value of the 200 ppm Sea Rose PW treatment group was 89% of the control fish FCE value for the final third of the study. Vignier et al. (1992) found reduced growth in salmon parr during a 40 day exposure to crude oil was not a consequence of reduced food intake and suggested the cause of lower FCE in treated fish could be a physical or physiological interruption of intestinal food uptake or alteration of metabolism. In contrast, the FCE value for the 100 ppm Venture PW treatment group was 30% higher than the control group in the final third of the our study. The FI for fish in the 2010 exposures was less than the FI in 2011 and 2012. The fish used in 2010 were initially smaller and from a different source than in 2011 and 2012. This size difference was unavoidable so that the 14 week exposure period from September to December was kept consistent between years.

Constraints on tank space and number of fish available for the studies required us to make a choice between replication of exposure concentration or testing at two different concentrations. We opted for two concentrations. While we cannot eliminate the possibility of tank effect, we note no differences in response of controls over several years of work.

PW is a highly complex mixture containing thousands of compounds with widely varying chemical properties. The complex nature of PW makes it difficult to try and relate biological effects to one or more specific compounds, and instead the toxicity of whole mixtures of chemicals should be considered, i.e. our data indicate the potential for effects to occur but can't address questions of what may cause the effects. The chemical composition of PW varies depending on the age, depth, and geochemistry of the hydrocarbon-bearing formation, as well as the chemical composition of the oil and gas phases found in the reservoir (Fisheries and Oceans Canada, 2011). In addition, the chemical characteristics may change after exposure to air resulting in each exposure being slightly different from previous ones under our conditions. This is unavoidable when an unlimited quantity of PW is not available.

The Venture and Thebaud platforms are both gas production wells in the Sable gas fields offshore of Nova Scotia, while Sea Rose and Terra Nova FPSOs are on oil fields in the Grand Banks offshore Newfoundland. As such, the chemical composition of PW between the two areas showed some significant differences. High concentrations of sulphur in Sea Rose and Terra Nova PW could indicate the presence of hydrogen sulphide, a known toxicant in PW (Sauer et al., 1992). Hydrogen sulfide may have formed by sulfate reducing bacteria over the long storage time of the PW in the storage containers but would have been minimal in the exposure tanks because of small volumes of the raw PW added (either 25 mL or 50 mL) to the 250 L of flowing seawater. Venture and Thebaud had concentrations of barium, iron, lead, manganese, strontium and zinc that were 2-3 orders of magnitude greater than Sea Rose and Terra Nova. Despite these high concentrations, dissolved metals likely play a minor role in the overall toxicity of PW in the natural environment (Neff, 2002). Dissolved metals in anoxic PW will precipitate on contact with oxygenated seawater (Stephenson et al., 1994), which then may settle on bottom sediments in the near-field zone around offshore oil and gas installations (Azetsu-Scott et al., 2007). Despite the high concentrations of BTEX, the high volatility of BTEX means that the exposure time would have been limited during this study. Lee et al. (2011) reported that aeration of PW (to simulate mixing with oxygenated seawater) resulted in an almost complete loss of BTEX after 25 hours. In addition, other studies have shown that the loss of BTEX does not significantly change the toxicity of PW to marine organisms (Flynn et al., 1996). Polycyclic aromatic hydrocarbons (PAHs) are the petroleum hydrocarbons of greatest environmental concern in PW because of their toxicity and persistence in the environment. Concentrations of PAHs were very similar between the four PW samples. The PAH profile in all four PW samples consisted predominately of low molecular weight compounds ($\leq$3 benzene rings), with 95-99% of the detected PAH compounds falling into this class. These compounds, such as naphthalene, fluorene, dibenzothiophene and phenanthrene are generally considered less toxic than high molecular weight PAH (Neff, 2002). In addition, aeration of the exposure water would have reduced the concentration of these semi-volatile compounds (Lee et al., 2011). Of greater concern with regard to toxicological effects are the high molecular weight PAHs with $\geq$4 benzene rings. These compounds were detected in the Venture, Sea Rose and Terra Nova PWs with total concentrations up to 0.03 mg/L, while they were not detected in the Thebaud sample. A wide range of phenolic compounds was also detected, with total phenol concentrations higher in the gas production well PWs versus the oil

production wells. Phenolic compounds, especially when present at high concentrations, may play a significant role in the toxicity of PW (Neff, 2002, Flynn et al., 1996). Straight chain saturated hydrocarbons (alkanes) were also detected in the four PWs. Alkanes are generally the result of small dispersed oil droplets in the PW (Neff, 2002), and are of minimal concern with regard to toxicity to marine organisms. Although the raw PW was not analysed at the end of the 14 week study and the treatment water in the exposure tanks was not analysed, we acknowledge that there are losses due to aeration, degradation and biological uptake (Binet et al., 2011; Burridge et al., 2011; Lee et al., 2011). These losses would happen naturally in the environment so there is no way to control them.

The negative effects on growth observed in this study are ecologically significant. These results suggest that juvenile cod are affected oy intermittent exposure. In the wild, depressed growth could result in size-dependent predation (Werner et al., 1983; Stephens et al., 2000). The effects of chronic, low level exposures of PW on important marine species such as cod may become evident only after monitoring several life stages. Earlier life stages (egg, larval and juvenile) of cod are vulnerable as they have little control over their movement in the ocean currents and may be unable to avoid drifting in and out of plumes or patches of PW and therefore may be exposed multiple times. Our results indicate that chronic intermittent long term exposure to environmentally relevant doses of PW had no effect on hepatic EROD activity of juvenile cod. The long term low dose exposure of juvenile cod to 100 ppm Thebaud PW and 200 ppm Sea Rose PW did affect SGR but the lack of a dose response in this study represents a challenge. The exposures of PW mimicked the rapid dilutions that occur following discharge into the environment. Discharge of PW to the waters of the Scotian Shelf and edge of the Grand Banks, two highly productive areas that have some of the highest abundance of cod in Canadian waters could pose a risk to juvenile cod. The data presented here indicate that effects can occur and need to be considered in a risk assessment context but generalizations about the effects of PW cannot be made. The complex nature of PW makes it difficult to try and relate biological effects to one or more specific compounds, and instead the toxicity of whole mixtures of chemicals should be considered. Chemical analysis of PW may be of little predictive value of toxicological effects. The consequences of long-term effects on cod populations from offshore oil and gas facilities that have a 15-20 year life-cycle need to be better understood.

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FIGURES

Figure 1. Locations of offshore oil and gas production sites for PW collection off the east coast of Canada. Stars indicate production sites.

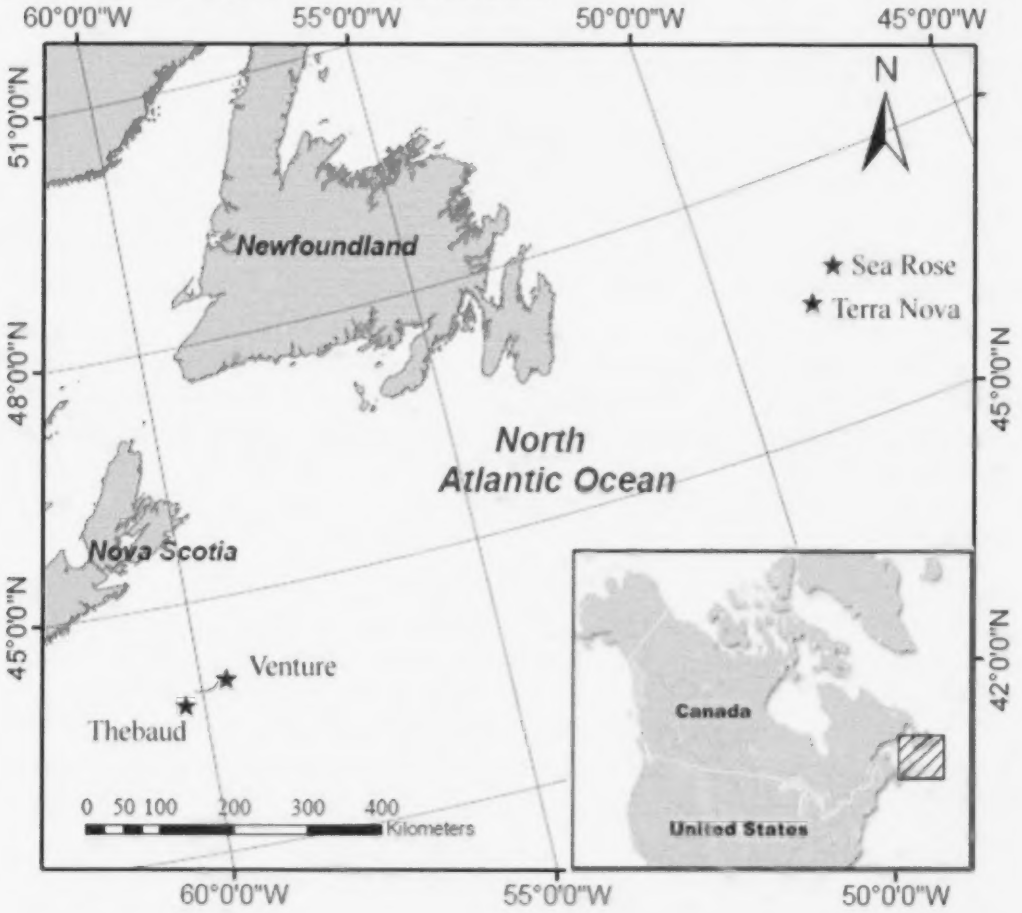
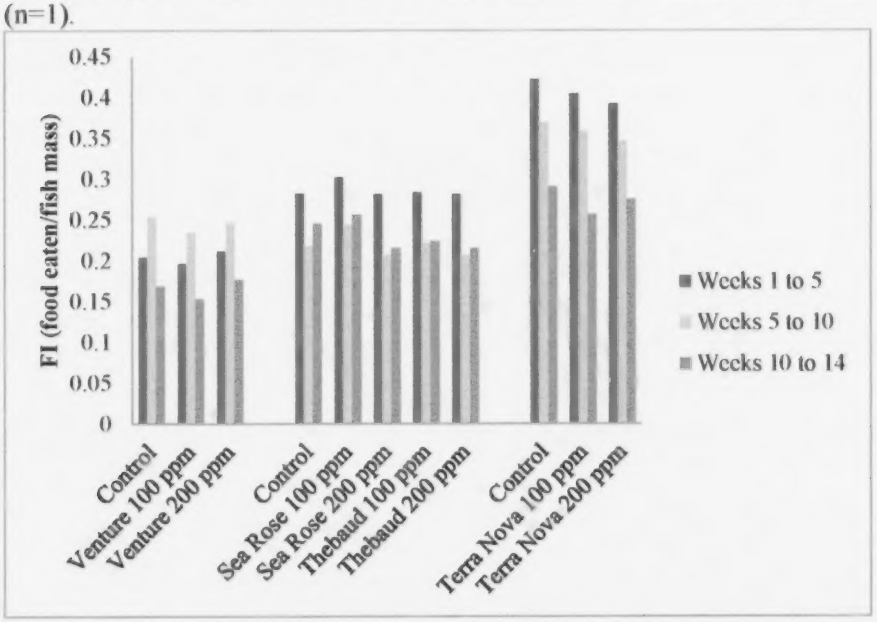


Figure 2. Feed intake of juvenile Atlantic cod at the three sampling times during the chronic exposure to PW. Values are total food eaten normalized for mass of fish in tank (n=1).



APPENDICES

APPENDIX 1.

Table 1. EROD activity in livers collected at sampling times from Atlantic cod chronically exposed to Venture PW, Sea Rose PW and Thebaud PW. Values are means ± S.E. (n=5 for treated fish and n=10 for control fish).

Treatment	EROD activity (pmol·min^{-1}·mg^{-1} protein)		
2010	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	3.583±0.638	3.806±0.690	2.873±0.540
Venture 100 ppm	2.059±0.113	5.934±1.644	3.878±0.773
Venture 200 ppm	1.687±0.425	6.621±2.699	6.846±2.736
2011	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	4.601±1.021	3.310±0.360	4.774±1.191
Sea Rose 100 ppm	3.473±1.217	4.187±0.945	5.161±1.489
Sea Rose 200 ppm	3.907±0.947	4.145±0.991	6.198±1.614
Thebaud 100 ppm	3.312±0.532	2.953±0.147	2.178±0.404
Thebaud 200 ppm	3.446±0.440	2.985±0.917	4.850±0.411

Table 2. Feed intake (n=1) and feed conversion efficiency (n=1) of Atlantic cod for weeks 10 to 14 of the chronic intermittent exposure to PW.

Treatment	Weeks 10 to 14	
	Feed intake	FCE
2010		
Control	0.169	0.847
Venture 100 ppm	0.153	1.101
Venture 200 ppm	0.177	0.785
2011		
Control	0.239	1.081
Sea Rose 100 ppm	0.256	1.062
Sea Rose 200 ppm	0.215	0.961
Thebaud 100 ppm	0.224	0.734
Thebaud 200 ppm	0.210	1.215
2012		
Control	0.290	0.836
Terra Nova 100 ppm	0.257	0.974
Terra Nova 200 ppm	0.275	0.936

APPENDIX 1.

Table 3. Mean specific growth rate ± SD of Atlantic cod. Asterisks indicate significant differences from control mean SGR (*P < 0.05, **P < 0.005) (Dunnett's test).

Treatment	Mean Specific Growth Rate		
2010	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	1.285±0.414 (n=68)	0.971±0.401 (n=55)	0.632±0.249 (n=41)
Venture 100 ppm	1.235±0.479 (n=36)	1.041±0.297 (n=26)	0.624±0.251 (n=17)
Venture 200 ppm	1.169±0.552 (n=26)	0.932±0.434 (n=29)	0.541±0.223 (n=23)
2011	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	0.906±0.508 (n=79)	0.912±0.347 (n=69)	0.809±0.258 (n=56)
Sea Rose 100 ppm	0.744±0.477 (n=39)	1.055±0.371 (n=30)	0.833±0.259 (n=23)
Sea Rose 200 ppm	0.921±0.350 (n=38)	0.848±0.298 (n=34)	0.612±0.271* (n=28)
Thebaud 100 ppm	0.758±0.533 (n=40)	0.949±0.324 (n=32)	0.550±0.363** (n=24)
Thebaud 200 ppm	0.881±0.375 (n=37)	0.962±0.318 (n=34)	0.804±0.246 (n=27)
2012	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	1.352±0.438 (n=76)	0.922±0.360 (n=71)	0.884±0.302 (n=61)
Terra Nova 100 ppm	1.246±0.441 (n=40)	0.967±0.354 (n=39)	0.904±0.430 (n=34)
Terra Nova 200 ppm	1.384±0.492 (n=40)	0.902±0.428 (n=38)	0.968±0.352 (n=33)

APPENDIX 2.

Table 1. Organic composition of PW from the four offshore platforms used in the study determined by GC-MSD.

Organic component	Concentration ($mg \cdot L^{-1}$)			
	Venture	Thebaud	Terra Nova	Sea Rose
BTEX	12.95	11.43	8.80	15.42
Alkanes	0.32	0.55	0.83	1.69
Alkylated PAH	0.30	0.47	0.26	0.53
Parent PAH	0.19	0.41	0.13	0.30
Phenols	10.62	16.54	4.04	3.52

APPENDIX 2.

Table 2. Summary of inorganic elements in PW.

Parameter	Concentration ($\mu g \cdot L^{-1}$)			
	Venture	Thebaud	Terra Nova	Sea Rose
Aluminum	100.0	180.0	< 50	< 50
Antimony	< 2	< 5	< 5	< 5
Arsenic	< 50	< 50	< 50	< 50
Barium	1240000	774000	430	860
Beryllium	1.2	1	0.3	0.3
Boron	29000	13300	32100	27000
Cadmium	2.40	1.07	< 0.02	< 0.02
Calcium	21800000	13600000	1050000	757000
Chromium	< 10	20.0	< 10	< 10
Cobalt	< 10	20	< 10	< 10
Copper	< 10	130	10	< 10
Iron	137000	146000	2980	5500
Lead	27	41	0.39	0.46
Lithium	36000	11700	2680	3980
Magnesium	1380000	1040000	426000	398000
Manganese	24100	19400	100	50
Mercury	0.1	0.15	0.011	0.013
Molybdenum	1.0	< 5	< 5	< 5
Nickel	< 20	140	< 20	< 20
Phosphorus	70	< 50	< 50	< 50
Potassium	1110000	685000	257000	287000
Rubidium	4400	1860	389	716
Selenium	< 50	< 50	< 50	< 50
Silicon	25600	14100	21800	24700
Silver	0.6	0.4	< 0.2	< 0.2
Sodium	495000000	430000000	15600000	10900000
Strontium	2410000	1450000	50800	67500
Sulfur	460	770	691000	549000
Tellurium	< 2	< 2	< 2	< 2
Thallium	140	70	< 2	4
Tin	< 0.5	< 0.5	< 0.5	< 0.5
Titanium	< 1	27	9	11
Uranium	< 0.005	< 0.005	< 0.005	< 0.005
Vanadium	< 5	9	< 5	< 5
Zinc	2400	2300	30	< 20